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CENTIGRADE

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THE EFFECT OF LOW TEMPERATURE ON THE SPIROCHETES OF RELAPSING FEVER

I. THE VIABILITY OF FOUR STRAINS OF SPIROCHETES STORED AT -48 DEGREES CENTIGRADE

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Among the very few organisms which cannot be maintained on the ordinary culture media are the spirochetes of relapsing fever. At present the methods widely used for the preservation of stock cultures of these agents are animal passage, chick embryo culture, infected ticks, and the freezing and subsequent storage at low temperature of infected material. The first three methods are expensive, time-consuming, and hazardous, and require special training; however, if cultures could be preserved at low temperature with assurance and a minimum of effort, the procedure would be a valuable aid to the bacteriologist.

The objective of this study was to develop a method for the preparation of suspensions of spirochetes that would retain their infectivity for mice after freezing and storage in a mechanical refrigerator at -48°C for long periods of time. In outlining the investigation, we directed particular attention to the stage of the disease at which the infected blood was secured and the medium in which the organisms were suspended for preservation.

Novy and Knapp (1906), in an exhaustive study of the morphology, viability, pathogenesis, cultivation, immunity, etc., of *Spirochaeta novyi* and related organisms, noted the survival of the forms in blood obtained during the onset of the infection; on one occasion a rat was infected with blood which had been stored for 37 days at room temperature. On the other hand, when the blood was obtained late in the course of the disease, the spirochetes were noninfective following storage for 2 days. They attributed the phenomenon to the appearance of a germicidal substance in the blood as the result of the multiplication of the organisms. The observation has been repeatedly confirmed by various workers in this laboratory, but no extensive investigation has been made of the basic mechanisms involved.

In recent years unusual interest has been devoted to the preservation of bacteria by freezing suspensions of the organisms and removing the water *in vacuo* while still in the frozen state. Excellent results have been obtained with various species of cocci and bacilli including such delicate forms as the meningococcus, gonococcus, and Pfeiffer's bacillus. Negative results were obtained by Turner (1938) when this method was employed for the preservation of *Spirochaeta pallida* and *Spirochaeta pertenuis*, and Oag (1939) had a similar experience with

¹ Based on data submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bacteriology, University of Michigan, November, 1944.

Spirochaeta duttoni. Swift (1921), many years earlier, had failed to preserve spirochetes in this manner and, in the course of his study, attributed the lack of survival of the organisms to the destruction by freezing and thawing.

In 1938 Jahnel cooled the blood and liver of infected rats to -269.3°C , and a typical spirochetosis followed the subsequent injection of the material into mice. Turner (1938) developed a procedure for the preservation of bacterial cultures at -78°C employing solid carbon dioxide and ethanol to obtain this temperature. The spirochetes of relapsing fever (Turner and Fleming, 1939) remained virulent under these conditions for relatively long periods of time; from a few weeks to a year. In a similar study Oag (1939) found that the spleen and liver of mice infected with *Spirochaeta duttoni*, frozen and stored at -78°C , maintained their infectivity for one month; but there was an attenuation of the virulence of the organisms proportional to the duration of the storage. This was manifested by a delay in the appearance of the spirochetes in the mice inoculated with the frozen material. In a study of the factors influencing the survival of organisms preserved by freezing and storage at low temperature, Turner and Brayton (1939) emphasized the importance of rapid thawing of the samples but found that variations in the freezing process had little influence on the infectivity of the spirochetes.

EXPERIMENTAL

The four strains of organisms used in these experiments were *S. novyi*, *S. obermeieri*, *S. duttoni*, and *S. kochi*, which were first observed in humans with relapsing fever in America, Europe, West Africa, and East Africa, respectively. They have been maintained in this laboratory for nearly 40 years by serial passage in white rats. The course of the disease in these animals is characterized by an incubation period of from 18 to 24 hours, following which the organisms appear in the blood of the peripheral circulation. They are clearly visible when the specimens are examined with a $\frac{1}{2}$ -inch oil immersion lens although the use of dark-field illumination makes them stand out more strikingly. Their numbers increase rapidly, and by the 4th day after inoculation, the blood may contain more organisms than red blood cells. From the 4th to the 5th day, there is a sudden decrease in the number of organisms in the blood. By the 6th day no forms can be found. A relapse may occur on or after the 7th day with the spirochetes reappearing in the blood stream of the infected rat.

The mechanical refrigerator used in all of the following work was a "deep-freeze"² specially designed to maintain a temperature of -48°C (± 3) uniformly throughout the storage cylinder. The infected specimens, either serum or blood, were distributed in cellophane tubes³ previously sterilized by ultraviolet irradiation. The selection of containers made of cellophane avoided the danger incident to the occasional breakage of glass tubes when subjected to repeated rapid changes in temperature. The organisms for the experiments were obtained by bleeding infected rats from the heart as follows: The rat was anes-

² Manufactured by the Motor Products Corporation, North Chicago, Ill.

³ Obtained from the Lusteroid Container Company Inc., South Orange, N. J.

thetized, the abdominal and thoracic cavities were opened aseptically, and the blood was drawn directly from the heart by means of a glass pipette (Novy and Knapp, 1906) and defibrinated by whipping with a glass rod. Albino rats weighing approximately 275 grams were used in order to obtain a large volume of blood. The presence of the spirochetes in the blood of white mice following the intraperitoneal injection of the samples in question indicated the survival of virulent organisms. No methods are available which indicate the viability of spirochetes in the absence of virulence.

In order to develop a method for the preparation of suspensions of organisms which would withstand freezing, storage at -48°C , and thawing, the following experiment was conducted. Heavily infected rat blood, drawn during the 4th day of the infection, was centrifuged at 1,700 rpm for 10 minutes to sediment the erythrocytes. By this process most of the spirochetes were concentrated in a white fluffy layer directly above the packed blood cells. Serum suspensions of organisms were obtained by the removal of the supernatant layer, including the fluffy layer, by means of a capillary pipette without disturbing the cells below. The suspension was thoroughly mixed and distributed in 0.2-ml amounts in cellophane tubes. The contents were prefrozen in an alcohol, solid carbon dioxide⁴ bath at -48°C and stored in the deep-freeze. Five days later representative samples were removed and thawed within 60 seconds in a water bath at 37°C . The suspensions were diluted 1:10 and 1:100 with nutrient broth and 0.1-ml amounts promptly injected into mice. Daily examinations of their blood were made for 11 days. The samples were obtained by the removal of a drop from the tail veins and observed with the oil immersion lens. None of the mice developed a spirochetosis. A second series of samples was removed after 10 days' storage, thawed as before, and diluted 1:5 with broth; 0.5-ml quantities were injected intraperitoneally into mice. No infections developed during the 7 days following the injection of the samples. A third group of tubes was removed after 14 days' storage, and the contents were thawed and injected undiluted into mice. All examinations failed to reveal the presence of organisms in the blood of the mice during the 4 days they were observed.

Serum suspensions of organisms from the 4th day after inoculation were prepared as before, distributed in 0.4-ml amounts, prefrozen, and placed in the deep-freeze. After 18 hours' storage representative samples, upon intraperitoneal injection into mice, failed to cause infections within 6 days.

Because of the failure of the previous work to give positive results, a preliminary experiment to investigate the value of using infected blood at earlier stages in the disease was planned. Spirochetosis was produced within 24 hours when samples, prepared from rats in the 3rd day of the infection, were treated in the same manner as in the preceding series and injected into mice. In this series inconsistent results were obtained when 0.4 ml of the frozen and stored samples, prepared from rats in the 2nd day of the disease, were injected into mice. Some developed a spirochetosis within 24 hours, whereas in others examination failed to reveal any organisms during the 9 days following examination. Subsequent

⁴ Commercial dry ice.

experiments showed this to be the result of the paucity of organisms in the material injected. In order to induce a heavy infection on the 2nd day, the rats

TABLE 1
The viability of spirochetes obtained during the 2nd day of the infection in rats and stored at -48 C

SERIES	VEHICLE	DAYS OF STORAGE	STRAIN OF ORGANISM			
			<i>S. novyi</i>	<i>S. obermeieri</i>	<i>S. duttoni</i>	<i>S. kochi</i>
1	serum	0	-8	-8	-8	-8
2		0	+3	+2	+2	+2
3*		42	-3	-3	+1	+1
		149	+2	+3	+4	+2
		223	-5	+5	-5	-5
		300	+1	-6	-3	+1
		800			+13	
3a*	defibr. blood	42	+1	+1	+1	+1
		149	+2	+1	+1	+1
		223	+2	+3	+1	+2
		300	+2	+4	+2	+3
		548	+1	-4	+3	+1
		800		+2	+1	
4		2	+1			+1
		4	+1			+1
5		2	+1			+1
		8	+2			+1
6		9	+1	+1	+1	
		40	+3	+2	+2	
		103	+2	+1	+2	
		166	+1	+2	+4	
		565	+1	-8	-8	
7		7	+1	+1	+1	
		38	+1	+1	+1	
		101	+1	+1	+1	
		164	+1	+2	+2	
		305	+5	+7	+6	
		563	-8	+5	+2	
		813	+9	+3	+1	

The presence of spirochetes in the blood of the mice is indicated by +; the absence, by -. The number of days after injection of the mice that the last examination was made is indicated by the numerals. The series 3a was a continuation of series 3, employing defibrinated blood in place of serum.

* Specimens were not frozen before being stored.

were given intraperitoneal injections of 0.75 to 1 ml of a saline suspension of heavily infected blood.

TABLE 2

The viability of spirochetes obtained during the 3rd day of the infection in rats and stored at -48 C

SERIES	VEHICLE	DAYS OF STORAGE	STRAIN OF ORGANISM			
			<i>S. novyi</i>	<i>S. obermeieri</i>	<i>S. duttoni</i>	<i>S. kochi</i>
1	serum	0	+3	+1	+1	+1
2		0	+1	+1	+1	+1
		2	-7	-7	-7	-7
3	defibr. blood	0	+1	+1	+1	+1
		2	-4	+7	-8	+3
4		1	+2	+1		+1
		7	+2	+2		+1
		17	+3	+2		+2
		598	-4	-4		-4
5	conc. susp.	6	+2	+3	+1	+2
		75	+1	+2	+2	+1
		390	+2	+2	+2	+1
		475	-8	+3	-8	+3
		694	+2	+1	+4	+1

See explanatory note to table 1.

TABLE 3

The viability of spirochetes obtained during the 4th day of the infection in rats and stored at -48 C

SERIES	VEHICLE	DAYS OF STORAGE	STRAIN OF ORGANISM			
			<i>S. novyi</i>	<i>S. obermeieri</i>	<i>S. duttoni</i>	<i>S. kochi</i>
1	serum	5	-11	-11	-11	-11
		10	-7	-7	-7	-7
		14	-3	-3	-3	-3
2		0	-6	-6	-6	-6
3	defibr. blood	2	-4	-4	-4	-4
		13	-3	-3	-3	-3

See explanatory note to table 1.

TABLE 4

The viability of spirochetes obtained during the 7th day of the infection in rats and stored at -48 C

SERIES	VEHICLE	DAYS OF STORAGE	STRAIN OF ORGANISM			
			<i>S. novyi</i>	<i>S. obermeieri</i>	<i>S. duttoni</i>	<i>S. kochi</i>
1	defibr. blood	2	+1			-4
		13	+2			-2
2		1	-3		-3	-3
		17	-3		-3	-3

See explanatory note to table 1.

On the basis of these preliminary findings, the survival after storage at low temperature of organisms from the progressive stages of the disease was studied. Infected rats were bled 2, 3, 4, and 7 days after inoculation. The spirochetes were prepared in (1) serum, as described before, (2) defibrinated blood, distributed without centrifugation, and (3) defibrinated blood, but in far greater numbers by the use of only the fluffy layer of organisms removed from the serum red cell interface following centrifugation. The samples were distributed in about 1-ml amounts, prefrozen as before, and stored in the deep-freeze. At various intervals the tubes were removed and the contents thawed rapidly and promptly injected into mice. The blood of these animals was examined daily for the presence of spirochetes. The results of the experiments are given in tables 1 through 4.

DISCUSSION AND CONCLUSIONS

The influence of storage at -48°C on the viability of four strains of the spirochetes of relapsing fever in the blood and serum of rats indicated that the organisms from early in the course of the disease retained their infectivity for mice for long periods of time. The stage of the disease at which the specimens were obtained was the most important factor in determining the length of time any sample remained infective. The spirochetes in blood removed on the 2nd day after inoculation were the most satisfactory for storage at low temperature. After 27 months at -48°C , these samples produced infections upon injection into white mice. Samples of blood taken on the 3rd day were infective following freezing and thawing. However, there was an increase in the incubation period of the disease in the mice that was in direct relation to the duration of storage. In contrast, specimens obtained during the 4th day were not infective after having been subjected to mere freezing and thawing. In those animals which showed a relapse, the number of organisms in the blood stream on the 7th day was somewhat comparable to the incidence in the 48-hour specimens. The attempts to preserve samples taken during a relapse gave inconsistent results. The latter finding may be due to the development of common antibodies during the first infection since it is postulated that the relapse phenomenon is caused by immunologically different strains which develop in the infected animal. No significant variations among the four strains were observed when subjected to this method of preservation.

A study was made in an attempt to determine the best way to prepare the specimens for storage at low temperature. Accordingly, three suspensions were used: defibrinated rat blood, serum, and the white fluffy layer which appeared at the interface between the cells and serum in centrifugated specimens of infected rat blood. The number of organisms present in these suspensions varied widely since, in the separation of the serum, many of the organisms in the defibrinated blood were trapped among the cells at the bottom of the tube, and the removal of the white fluffy layer only resulted in a small volume of blood containing great numbers of spirochetes. Infected rats were bled on the 3rd day since the organisms at this stage exhibited a rapid decline in infectivity

during the storage period, which would demonstrate marked differences, if such existed, among the three suspensions. The results of the experiment showed conclusively that the number of organisms in the specimen was of greater significance than the type of preparation. As an example, serum suspensions failed to produce an infection after only 2 days' storage, defibrinated blood suspensions were infective after 17 days but not after 598 days, but the concentrated suspensions were highly infective after 694 days' storage.

When the suspensions of organisms were frozen, stored, and thawed, many of the cells retained their spiral morphology; however, some were distinctly granular, and many did not regain their motility. The bizarre and fragmented forms observed in these specimens would indicate that the decrease in the numbers of organisms following freezing and thawing resulted from the destruction of the spirochetes. This was more marked in the samples of blood from the later stages of the infection.

SUMMARY

A simple method has been developed for the preservation of the spirochetes of relapsing fever at low temperature. Samples of rat blood containing *Spirochaeta novyi*, *Spirochaeta obermeieri*, *Spirochaeta duttoni*, and *Spirochaeta kochi*, obtained 48 hours after inoculation, remained infective for mice following 27 months of storage in a mechanical refrigerator at -48°C . The stage of the disease during which the infected blood was obtained was the most important factor in determining the survival of the organisms. However, the number of organisms present in the specimens also appeared to be significant in limiting the period of time any sample would retain its infectivity.

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